

Report

Amorphas Silica Nanoparticles Decrease Human Chorionic Gonadotropin β Expression During Syncytialization of BeWo Cell

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Nanoparticles are used in many everyday products because of their innovative properties, but there are concerns that, being smaller than conventional materials, they have the potential to induce unexpected biological effects. Despite the need for the safety of nanoparticles to be assessed, action in this area is still lagging. Several studies, including those by us, have revealed that nanoparticles can reach the placenta and show placental toxicity, but it remains unclear how nanoparticles affect placental function. Here, we attempted to assess the effect of nanoparticles on placental hormone-producing function by using an *in vitro* forskolin-induced BeWo syncytialization model. BeWo cells were treated with amorphous silica nanoparticles with a diameter of 10 nm (nSP10) either during syncytialization or after being syncytialized by forskolin treatment. RT-PCR analysis showed that nSP10 inhibited forskolin-induced upregulation in the expression level of human chorionic gonadotropin β (hCG β ; gene name, *CGB*) during syncytialization, but not in syncytialized BeWo cells. Moreover, nSP10 down-regulated forskolin-induced elevation in the expression of *endogenous retrovirus group FRD member 1 (ERV-FRD-1)* and *syndecan-1 (SDCI)*, which are involved in cell fusion during syncytialization. These results suggest that nSP10 could suppress trophoblast cell fusion, thus inhibiting the production of hCG in syncytialized BeWo cells.

Key words human chorionic gonadotropin β , placenta, silica nanoparticle, syncytialization

INTRODUCTION

Because the placenta facilitates delivery of oxygen and nutrients and removal of waste products between mother and fetus, impairment of the placental structure and the resulting dysfunction leads to restricted fetal growth.¹⁾ Therefore, normal placental maintenance and development is essential for sustaining the growth of the fetus. The outermost layer of the villi that make up the placenta is covered with syncytiotrophoblast cells, which are formed by the differentiation/fusion (syncytialization) of cytotrophoblast cells localized inside the villi.²⁾ This layer of syncytiotrophoblasts not only functions as a blood–placental barrier but also produces hormones, such as human chorionic gonadotropin (hCG), that perform multiple functions in the development of the placenta and fetus.³⁾ Because hCG is a pregnancy-specific hormone produced by the syncytiotrophoblasts, its concentration is a marker for the subsequent clinical manifestation of preeclampsia.⁴⁾ Therefore, the effects of environmental factors on syncytiotrophoblast cells and syncytiotrophoblast formation could impact hormone production in the placenta and subsequent growth of the fetus.

In recent years, the development and use of nanoparticles have been expanding.⁵⁾ A characteristic function of nanoparticles is their greater specific surface area than that provided by conventional particles, and nanoparticles have come to be

utilized in daily necessities and pharmaceuticals. For example, silica nanoparticles are frequently and repeatedly used as components of daily products such as cosmetics and foods.⁶⁾ Thus, the risk of exposure to nanoparticles occurs regardless of age or gender.⁷⁾ On the other hand, the collection of hazard information, which is essential for understanding the risk of nanoparticles, is inadequate, and particularly the lack of information worldwide on pregnant mothers has been noted.⁸⁾ In this regard, we previously showed that the difference from conventional materials in the physical properties of silica nanoparticles induces collapse of the placental structure by damaging the placental trophoblast layer and retarding intrauterine growth.^{9,10)} Therefore, we assessed the effect of silica nanoparticles on placental function in terms of hormone production in syncytiotrophoblast cells and during syncytiotrophoblast formation.

MATERIALS AND METHODS

Cell Line and Cell Culture The human choriocarcinoma cell line BeWo was purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB9111; Osaka, Japan). BeWo cells were cultured in 10% inactivated fetal bovine serum (Biosera, Nuaille, France) and 1% (v/v) penicillin–streptomycin–amphotericin B suspension (Wako, Osaka, Japan) in Ham's F-12 nutrient mixture (Wako). BeWo cells were maintained

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under standard cell-culture conditions at 37°C and > 95% humidity in a 5% CO₂ atmosphere.

Reagents An aqueous suspension of silica nanoparticles with a diameter of 10 nm (nSP10) was purchased from Micromod Partikeltechnologie (Rostock, Warnemünde, Germany). Before use in experiments, nSP10 was sonicated for 5 min at 400 W (Ultrasonic Cleaner, AS ONE, Osaka, Japan) and mixed for 1 min with a benchtop vortex mixer (Digital VORTEX-GENIE 2, Scientific Industries Inc., New York, USA). Forskolin and H-89 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Forskolin was diluted with Ham's F-12 to a final concentration of 50 µM, and H-89 was diluted with Ham's F-12 to a final concentration of 10 µM.

Cell Viability Assay BeWo cells were seeded at 1.0×10^4 cells/200 µL per well into a 96-well, flat, clear plate. To assess the effect of nSP10 on syncytialized trophoblasts, BeWo cells were pre-treated with 50 µM forskolin for 24 h. After removing forskolin, BeWo were treated with nSP10 (12.5, 25, 50, 100, or 200 µg/mL) for 72 h. Also, to assess the effect of nSP10 on trophoblasts during the syncytialization process, BeWo cells were treated with 50 µM forskolin and nSP10 (3.12, 12.5, 50, 100, 200, or 400 µg/mL) for 48 h. After treatment, cell viability was measured with the colorimetric dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Tokyo Chemical Industry, Tokyo, Japan) in accordance with the manufacturer's instructions. Viability was determined with respect to the control (i.e. cells untreated with nSP10).

RT-PCR Analysis BeWo cells were seeded at 1.0×10^5 cells/1 mL per well in 12-well, flat plates, pre-treated with 50 µM forskolin for 24 h, and then treated with nSP10 (12.5, 25, or 50 µg/mL) for 72 h. To assess the effect of co-treatment of nSP10 on the syncytialization process, BeWo cells were seeded at 1.5×10^5 cells/2 mL per well in 6-well, flat plates and treated with 50 µM forskolin and nSP10 (3.12, 12.5, or 50 µg/mL) for 48 h. H-89 was used as a positive control to suppress syncytialization. Total RNA was extracted by using a FastGene RNA Kit (Nippon Genetics, Tokyo, Japan) and reverse-transcribed into cDNA by using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). A PCR mixture was prepared containing the above cDNA as a template and primers for the genes, *CGB* (forward, 5'-GCTCACCCAGCATCCTATC-3' and reverse, 5'-CCTGGAACATCTCCATCCTTG-3'), *endogenous retrovirus group FRD member 1 (ERVFRD-1)* (forward, 5'-GCAGCACAGGGAGGAATTTG-3' and reverse, 5'-TGGCTCGTTCCCGTAAACTG-3'), *syndecan-1 (SDC1)* (forward, 5'-AGCACTTACTGGTAGGACCAAGC-3' and reverse, 5'-TTCTTAACCCTGATGCTGTCTCC-3'), and *actin* (forward, 5'-GCCCTGAGGCACACTTCCA-3' and reverse, 5'-CGGATGTCCACGTCACACTTC-3') (Eurofins Genomics, Tokyo, Japan), as well as GeneAce SYBR qPCR Mix α Low ROX (Nippon Gene, Tokyo, Japan). RT-PCR was performed by using a CFX-384 Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA, USA). The expression level of each gene was normalized to that of *β-actin*.

Statistical Analysis Statistical analyses were conducted by using GraphPad Prism (version 9.3.1). Data are expressed as means ± S.D. For the results in Fig. 1, statistical analyses were performed using Dunnett's method. For the results in Fig. 2 and Fig. 3, statistical analyses were performed using Tukey's method. *P*-values lower than 0.05 were considered

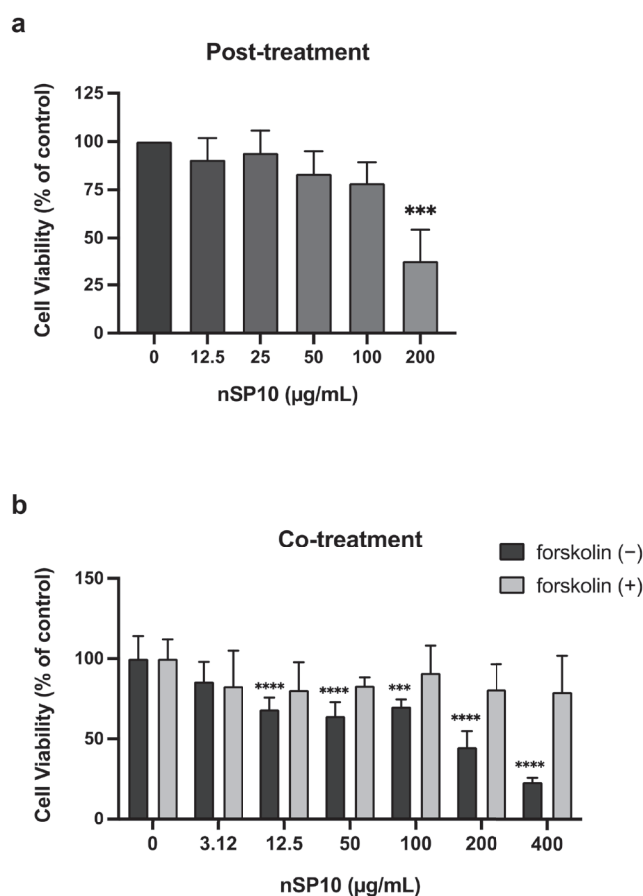


Fig. 1. Cytotoxicity of Amorphous Silica Nanoparticles in Human Chorionicarcinoma BeWo Cells

(a) BeWo cells were pre-treated with forskolin for 24 h and then treated with amorphous silica nanoparticles (nSP10) for 72 h. (b) BeWo cells were co-treated with forskolin and nSP10 for 48 h. Cell viability was evaluated by MTT assay and was determined with respect to the control (i.e. cells untreated with nSP10). Independent experiments were performed two times. Data are expressed as means ± S.D., $n = 5$. *** $P < 0.001$, **** $P < 0.0001$ versus value for groups untreated with nSP10.

statistically significant.

RESULTS AND DISCUSSION

nSP10 Suppressed *CGB* Expression in BeWo Cells During Syncytialization hCGβ, governed by the *CGB* gene, is a hormone mainly synthesized by syncytiotrophoblast cells in the placenta and plays a critical role in stimulating the production of progesterone, which is necessary for maintaining normal pregnancy in the early stage.¹¹⁾ For the *in vitro* model, we used BeWo trophoblasts cells, which are commonly used to study the dynamics of cytotrophoblast differentiation into syncytiotrophoblasts¹²⁾ and as a cell model for hCGβ production by trophoblast cells.¹³⁾ The cell viability assay showed that, at a dose of 50 µg/mL, nSP10 was not toxic to pre-syncytialized BeWo cells (Fig. 1a). In contrast, nSP10 co-treated with forskolin was not toxic to BeWo cells during the syncytialization process at this condition (Fig. 1b). We then analyzed the expression of *CGB* mRNA. RT-PCR analysis showed that there was no significant change in *CGB* expression level at each dose of nSP10 in pre-syncytialized BeWo cells (Fig. 2a). However, co-treatment with forskolin and nSP10 (50 µg/mL) significantly

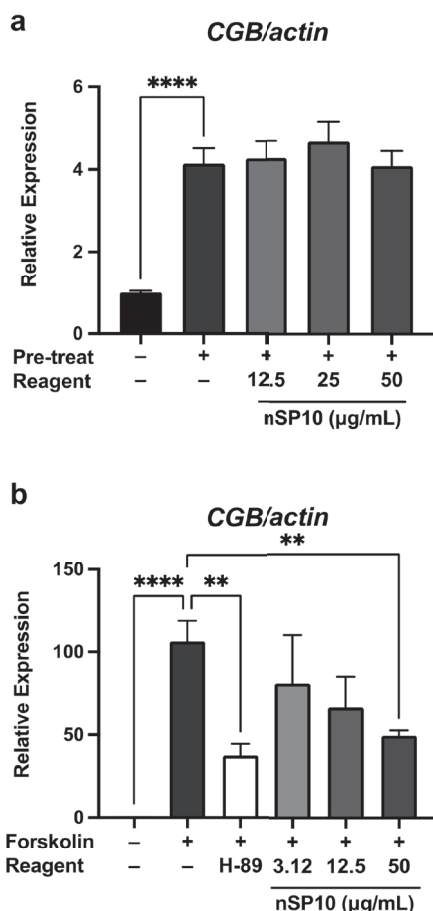


Fig. 2. Amorphous Silica Nanoparticles Suppressed *CGB* Expression in Human Choriocarcinoma BeWo Cells During Syncytialization

The expression of *CGB* mRNA (a) in BeWo cells treated with amorphous silica nanoparticles (nSP10) after syncytialization and (b) in BeWo cells treated with nSP10 during the syncytialization process was analyzed by RT-PCR. The relative expression level of *CGB* was analyzed after normalization to that of *actin*. Data are presented as means ± S.D. of three independent experiments: ***P* < 0.01, *****P* < 0.0001.

decreased the forskolin-induced upregulation in *CGB* expression level (Fig. 2b). At this time, the expression of *CGB* was significantly decreased by H-89 (a protein kinase A inhibitor used as a positive control for inhibition of the syncytialization process).¹⁴ These results suggest that nSP10 has the potential to inhibit placental hormone production during syncytiotrophoblast formation.

One of the reasons for the decrease in *CGB* expression during co-treatment may be a difference in the uptake pathway. A previous study reported that trophoblasts actively uptake amino acids through activation of macropinocytosis as syncytialization progresses.¹⁵ Activation of this mechanism may make trophoblasts sensitive to foreign substances during syncytialization. In addition, it has been shown that some nanoparticles are taken up into cells by macropinocytosis.¹⁶ Indeed, in this study nSP10 induced strong cytotoxicity against BeWo cells that were undergoing syncytialization compared with previously syncytialized BeWo cells. Therefore, we are currently using endocytosis inhibitors to analyze the effect of differential nSP10 uptake into BeWo cells.

Since other placental hormones are produced by syncytiotrophoblasts along with hCGβ, nSP10 may affect the produc-

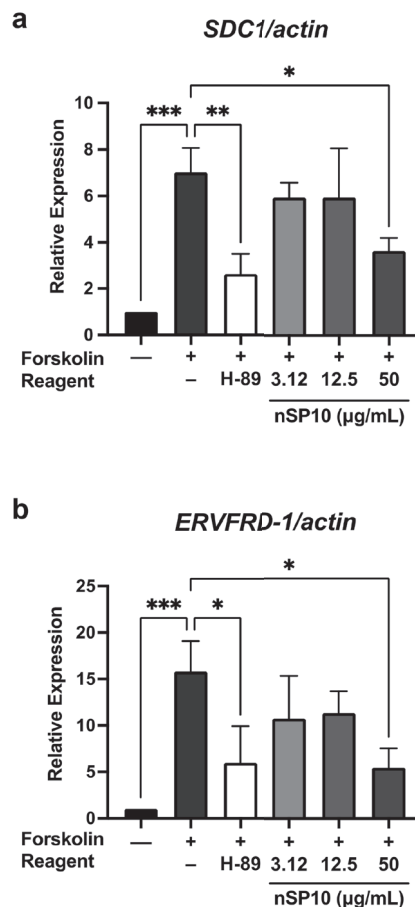


Fig. 3. Amorphous Silica Nanoparticles Suppressed Forskolin-Induced BeWo Syncytialization

Human choriocarcinoma BeWo cells were treated for 48 h with forskolin alone or with forskolin containing amorphous silica nanoparticles (nSP10) or H-89, as a positive control to suppress syncytialization. The expression of (a) *SDC1* and (b) *ERVFRD-1* mRNA was analyzed by RT-PCR. The relative expression level of each gene was analyzed after normalization to that of *actin*. Data are presented as means ± S.D. of three independent experiments: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

tion of these hormones during syncytialization. For instance, *fms*-like tyrosine kinase 1 promotes placental angiogenesis¹⁷ and human placental lactogen is involved in maternal glucose metabolism.¹⁸ It is necessary in the future to use these hormones as an index to evaluate the effects of nSP10 on syncytiotrophoblasts.

nSP10 Suppresses Forskolin-Induced BeWo Syncytialization Given that many of the functions in the placenta are maintained mainly by syncytiotrophoblasts, the process of syncytiotrophoblast formation, in which cytotrophoblast cells fuse and differentiate into multinucleated syncytiotrophoblast cells, is important throughout pregnancy.^{19,20} Thus, to examine the effect of nSP10 on forskolin-induced syncytialization in BeWo cells, we evaluated the mRNA expression of *ERVFRD-1* (which encodes syncytin-2) and *SDC1* (which encodes syndecan-1), because both genes play important roles in cell fusion and signaling during the trophoblast syncytialization process.²¹ Actually, we confirmed that the forskolin-induced elevation level of both genes was significantly decreased by H-89. RT-PCR analysis showed that co-treatment with nSP10 (50 µg/mL) and forskolin resulted in a significant decreased *SDC1* (Fig. 3a) and *ERVFRD-1* mRNA levels (Fig. 3b). These results suggest that

nSP10 decreased the expression of *ERVFRD-1* and *SDCI* during trophoblast formation, thus suppressing the production of hCG β in BeWo cells during syncytialization.

Syncytin-2 is highly expressed in mononuclear trophoblast cells and plays important roles in each step of the mononuclear trophoblast cell fusion process.²²⁾ Thus, syncytin-2 is essential for placental and fetal development in early pregnancy. Aberrant expression of the mediating gene adversely affects the pregnancy outcome, as evidenced by embryonic lethality of all fetuses in *syncytin-A* knockout mice.²³⁾ Our result suggests that nSP10 could suppress the mononuclear fusion process via downregulation of *ERVFRD-1* expression. Inadequate syncytialization can also affect pregnancy outcomes; therefore, we are currently other studies to examine the effects of nSP10 on pregnant mice.

Syndecan-1 is a transmembrane proteoglycan with increased expression during syncytialization and is responsible for cell signaling through binding to growth factors such as transforming growth factor- β and through interaction with the extracellular matrix.²⁴⁾ Therefore, alterations in *SDCI* expression are known to lead to abnormal placental function, and indeed decreased levels of syndecan-1 in the serum of preeclampsia patients have been reported.²⁵⁾ Previous studies in the BeWo model have also shown that hCG β production is reduced when syndecan-1 is silenced by siRNA.²⁶⁾ From the above, nSP10 may induce decreased placental function by inhibiting hCG β production via downregulation of *SDCI*.

Collectively, we revealed that nSP10 could suppress trophoblast cell fusion, thus inhibiting the production of hCG in syncytialized BeWo cells. Our current findings suggest that nSP10 during syncytialization process may induce a decline in placental function. Therefore, further studies are needed to understand the extent to which nSP10-induced hCG β reduction affects pregnancy outcome.

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Conflict of interest The authors declare no conflict of interest.

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